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Purification and kinetics of uricase from fenugreek

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Abstract: Uricase (EC 1.7.3.3) is an enzyme involved in the purine breakdown pathway. The enzyme was isolated from fenugreek (*Trigonella foenum-graecum*) leaves and purified by 80% ammonium sulfate, DEAE-cellulose and Sephadex G-200. The final specific activity was 230.76 Umg^{-1} protein and the final yield of purification was 2.87 % with 133.38-fold. The optimal pH and the optimal temperature were 8.0 and 40°C. The enzyme was inactivated by the chelating agent as α-α-dipyridyl and the IC_{50} value of α-α-dipyridyl was 9.03 mM.

keywords: Uricase, Fenugreek (*Trigonella foenum-graecum L.*), Purification, Characterization.

1.Introduction

Enzymes are very specific molecules for their action on their substrates. They are synthesized by living cells [1]. Enzymes accelerate the chemical reactions in the cell without destroying it during the reaction and are used over and over. The substance, which is converted into a product by enzymes, is called enzyme substrate [2]. The living cell contains several types of enzymes; each enzyme is specific to a certain chemical reaction [3, 4].

Uric acid is present in blood plasma as an end result of purine metabolism and also forms in the body, resulting in tumor lysis syndrome, wherein the malignant cells burst and abruptly release nucleic acids, proteins, and other metabolites into the blood stream, which leads to hyperuricemia [5, 6].

The lack of functional uricase and excretion of uric acid leads to various health problems, such as gout, hyperuricemia, cardiovascular risk, as well as renal failure [7, 8, 9].

Generally, the normal concentration of uric acid in serum is in the range of 0.15–0.42 mM [10, 11] and 1.49–4.46 mM [12] in urinary excretion. A uric acid value that exceeds the normal range may lead to various diseases such as hypertension, gout, diabetes, hypertriglyceridemia, metabolic syndrome, coronary heart disease, and kidney damage [13, 14, 15, 16].

Uricase (EC1.7.3.3) is one of the oxidoreductase which is the first group of enzymes [17]. Uricase is an enzyme involved in the purine breakdown pathway. In the presence of oxygen, uricase stimulates uric acid oxidation to hydrogen peroxide and allantoin, which acts as an electron acceptor [18]. Allantoin is more soluble and more easily excreted than the starting compound by the kidney in most prokaryotic and eukaryotic organisms [19].

$$\text{Uric acid} + O_2 + H_2O \rightarrow \text{Allantoin} + H_2O_2 + CO_2$$

Although humans are unable to create uricase due to a mutation in the fifth exon of the uricase gene, microorganisms, animals, and higher plants can produce uricase on their own [20, 21, 22, 23]. In higher plants, purine degradation has been studied, especially in legumes that grow symbiotically with nitrogen-fixing bacteria. This degradation produces ureides, or allantoic acid, and allantoin, which are important for nitrogen mobilization in many nodulated seedlings [24].

The aim of the present work aimed to isolate, purify uricase enzyme from a medicinal plant fenugreek and study its biochemical properties.

2. Materials and methods

Enzyme extraction

Leaves (10 gm) of *Trigonella foenum-graecum L.* (family: Fabaceae) were suspended in 50 ml of prechilled phosphate buffer (50 mM) at pH 8.0, and the mixture was blended in a Warren blender and stirred. After that, the homogenate was centrifuged for ten minutes at 10,000 rpm. The crude enzyme extract that resulted from the supernatant was used for further analysis [25].

Uricase assay

By using the method of Suzuki (1981), the activity of uricase was determined. The standard mixture of reactions included 1.5% phenol, 0.15 ml of 30-mM 4-aminoantipyrine, 0.05 ml of 15 U/ml peroxidase preparations, 0.1 ml of enzyme solution, and 0.6 ml of 2 mM uric acid diluted in 0.1 M sodium borate buffer (pH 8.0). For 20 min the mixture was incubated at 40°C. Then 1 ml of ethanol was added to stop the reaction, and by spectrophotometer, the absorbance at 540 nm was recorded against the blank. With the standard assay, a single unit of uricase was described as the quantity of enzyme that releases one μmol of H_2O_2 per min.

Purification of uricase

Three procedures were used to purify uricase, including:

1- Ammonium sulfate precipitation By adding various concentrations of solid ammonium sulfate to the crude extract, the precipitation step was completed. After slowly adding 80% (v/v) of solid ammonium sulfate and mixing for 30 minutes at 4°C, the mixture was centrifuged for 30 minutes at 4°C at 8000 rpm. The precipitate was dissolved in a tiny volume of borate buffer (100 mM) at pH 8.0, dialyzed against 500 ml of the same buffer for 24 hours, and then concentrated with sugar. Protein concentration and uricase activity were measured [27].

2- Ion exchange chromatography on DEAE-cellulose A 2.0 x 60 cm glass column was loaded with DEAE-cellulose. The purified and filtered cell-free supernatant was added to a column that had already been stabilized using 100 mM borate buffer (pH 8.0). The column was rinsed with 3 times amounts of 100 mM borate buffer, pH 8.0, at a velocity of 40 ml/h, and by using a fraction collector and analyzing the bound proteins by UV spectrophotometer at 280 nm, the bound proteins were released with a straight NaCl gradient (100 mM) in the same buffer. Polyethylene glycol was used to collect and concentrate fractions containing the uricase enzyme [28, 29].

3- Gel filtration column (Sephadex G-200)The enzyme, which was collected from DEAE-Cellulose, was applied to Sephadex G-200 (2x 60 cm) equilibrated with 0.2 M Tris-HC1 (pH 8.0). A gradual flow rate of 0.6 ml/min was used to ensure the best separation of the enzyme from other proteins. The fractions that contain uricase activity were combined and concentrated using polyethylene glycol (PEG). The purification of uricase was confirmed by SDS-PAGE (data not shown).

Determination of total soluble protein

By Bradford (1976), the content of soluble protein in the extract was determined [30].

Effect of pH on the activity of purified uricase

Studying the effect of pH on uricase activity was carried out at various pH. Glycine-HCl (100 mM) was used for pH 2–3. Phosphate buffer was used for pH 6.0–8.0, while borate buffer was utilized for pH 8.0–10.0. Then uricase activity was measured, as mentioned above.

Effect of temperature on the activity of purified uricase

The optimal temperature of the uricase reaction was determined by incubating uricase at various temperatures (10 - 60°C). Then uricase activity was measured, as mentioned above.

Effect of different concentrations of purified uricase on its activity

Studying the effect of uricase concentration on its activity was carried out at various uricase concentrations (0.2–1.0 mg protein). Mix well and leave for 15 min, then stop the reaction with one ml of 80% (v/v) ethanol, and the uricase activity was calculated.

Effect of α-α-dipyridyl on the activity of purified uricase

The effect of α-α-dipyridyl as a chelating agent was examined at various concentrations (2–10 mM) in the reaction medium, and then the uricase activity was determined.

3. Results and Discussion

Uricase is one of the few enzymes with high therapeutic values. The results of uricase purification are shown in Table 1. These results indicate that the specific activity of the enzyme increased progressively from the crude enzyme extract to Sephadex G-200. The final specific activity of Sephadex G-200 was 230.76 Umg-1 protein. The enzyme was purified 133.38-fold with a yield of 2.87 %.

Enzyme purification for industrial applications requires a simple and quick procedure to be useful and cost-effective [31]. One aim of the current study was to purify the uricase from fenugreek leaves. The purified enzyme is needed for specified biochemical analyses to permit a greater understanding of enzyme catalytic mechanisms.

Production of enzymes is essential for industrial sectors because of the great and high performance of enzymes from various sources, which act fine under a broad range of different chemical and physical conditions.

Table 1: Purification schedule of uricase.

Purification step	Protein content (mg)	Total activity (U)	Specific activity (Umg^{-1} protein)	Purification fold	Yield (%)
Crude extract	600	1043	1.73	1	100
Ammonium sulphate (80%)	105	846	8.05	4.65	81.11
DEAE-cellulose	11	266	24.18	13.97	25.5
Sephadex G-200	0.13	30	230.76	133.38	2.87

Uricase activity was measured in a pH range of 2 to 10. The results in Fig. 1 indicate that the optimal pH of uricase was 8, and the activity declined progressively after the optimal pH.

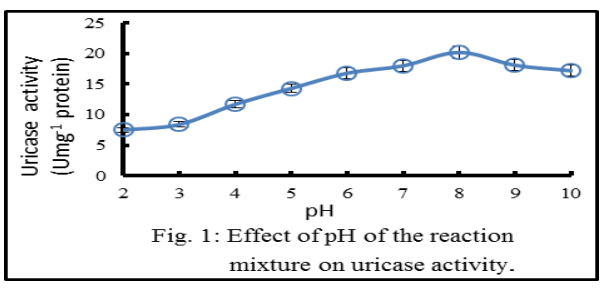

Fig. 1: Effect of pH of the reaction mixture on uricase activity.

Since the ionizable side of amino acid chains has properties that are dependent on pH, changes in pH typically cause variations in the enzyme activity. Proteins are denatured and lose their tertiary structure at high pH levels. Enzyme activity may be dependent on the ionization degree of specific amino acid chains; even at moderate pH values where the tertiary structure is not disturbed [32]. An enzyme's pH profile may reveal the identity of those residues. The following are possible outcomes of an extremely high pH. Firstly, it modifies the enzyme-substrate complex's ionization. Secondly, it modifies the ionization of different groups within the enzyme molecule, potentially influencing the enzyme's substrate affinity. Thirdly, it modifies the substrate's ionization, which may affect how the substrate binds to the enzyme. Fourthly, it has the ability to modify protein structure at an extremely high pH, which changes irreversibly the stability of the enzyme [33].

Uricase activity was studied at different temperatures (10- 60 °C). The results in Fig. 2 reveal that the uricase activity was increased with increasing the temperature up to 40°C and this is considered as the optimal temperature.

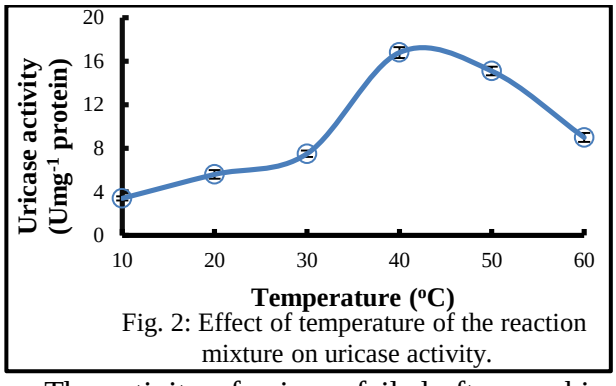

Fig. 2: Effect of temperature of the reaction mixture on uricase activity.

The activity of uricase failed after reaching the optimal temperature. Rising the temperature causes an enhancement in the inherent energy of the system. Thus, more molecules can obtain the essential activation energy for the reaction because the influence of temperature on the activation of the molecules is equal to the diminution of the reaction because of the damage to the tertiary structure [33, 34, 35]. At this point, the activity is at its maximum, and this temperature is frequently called the optimum temperature [36]. Changing in the temperature of the protein alter the degree of vibrational motion and diffusion of protein molecules that effects on protein composition

and aggregation [37]. Enzymes are usually quite stable at the temperature ambient for the organism from which they are obtained and lose their activity when the temperature is increased to a significantly higher level.

The effect of different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg protein) of uricase on its activity was investigated. The results in Fig. 3 show an increase in uricase activity with an increase in its concentration.

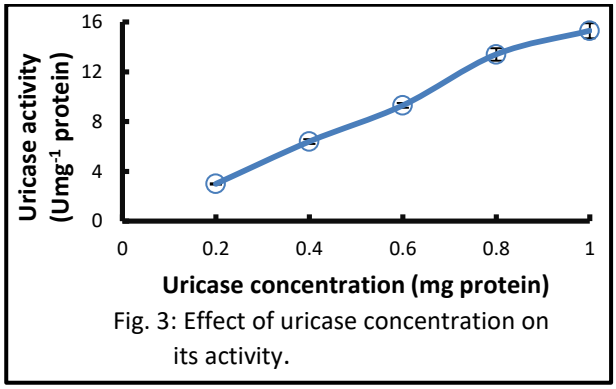

Fig. 3: Effect of uricase concentration on its activity.

α-α-dipyridyl as chelating agent was tested at 2, 4, 6, 8 and 10 mM in the reaction mixture (Fig. 4).

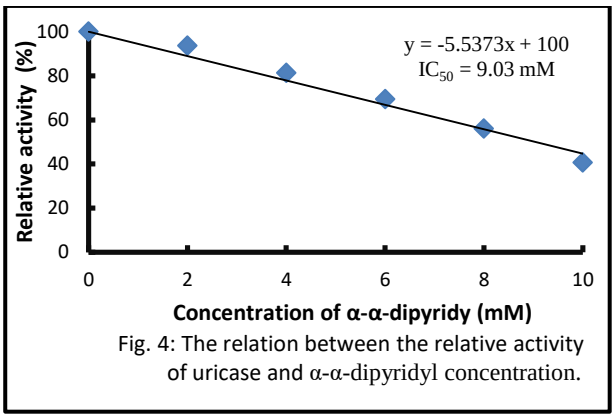

Fig. 4: The relation between the relative activity of uricase and α-α-dipyridyl concentration.

The results show that α-α-dipyridyl inactivated uricase, and inactivation was dependent on its concentration. The IC_{50} value of α-α-dipyridyl was 9.03 mM. α-α-dipyridyl is a chelating agent for enzymes [38]. This inhibition of uricase by α-α-dipyridyl indicates that the enzyme is a metalloenzyme.

In conclusion

Uricase was successfully purified from fenugreek leaves and showed an optimal temperature of 40°C and an optimal pH of 8.0. It is also a metalloenzyme.

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